Chapter 4

Low retinol levels potentiate bile salt-induced expression of the bile salt export pump in vitro and in vivo

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4.1 Abstract

The farnesoid X receptor/retinoid X receptor α (FXR/RXRα) heterodimer regulates transcription of genes involved in bile acid homeostasis, including the *Bile Salt Export Pump* (*BSEP*) and *Small Heterodimer Partner 1*(*SHP*). FXR is activated by bile acids and RXRα by 9-cis retinoic acid (9cRA). We evaluated the role of 9cRA in the expression of *BSEP*/*Bsep* and *SHP*/*Shp in vitro* and *in vivo*.

Human *BSEP* and *SHP* expression was quantified by real time RT-PCR in HepG2-rNtcp cells with or without transfection of rFxr- and hRXRα-expression plasmids and cultured in the presence or absence of chenodeoxycholic acid (CDCA) and/or 9cRA. *BSEP* promoter activity was measured by luciferase reporter assays and FXR/RXR DNA binding by electrophoresis mobility shift assays. Vitamin A-depleted C57BL/6J mice were used to evaluate the effect on cholic acid-induced *Bsep* and *Shp* expression *in vivo*.

In vitro, 9cRA strongly antagonized the CDCA-dependent *BSEP* gene transcription, by inhibiting binding of the FXR/RXR heterodimer to the *BSEP* FXR response element. In contrast, 9cRA agonized *SHP* expression. *In vivo*, vitamin A depletion enhanced cholic acid-induced expression of *Bsep* mRNA and protein, while reducing *SHP* expression.

9cRA either agonizes (*SHP*) or antagonizes (*BSEP*) bile acid-activated transcription of FXR/RXR-target genes. Vitamin A is therefore an important determinant in regulation of bile acid transport and synthesis. In patients with obstructive cholestasis, vitamin A derivatives may be therapeutically useful to decrease *BSEP* expression, thereby reducing the hepatobiliary bile acid flux and as a result reduce the pressure in the biliary tree.

4.2 Introduction

Vitamin A or retinol is important in a number of biological processes, including reproduction, embryogenesis, visual function, growth and development. All vitamin A present in the body is acquired from the diet. It is mainly stored in the stellate cells of the liver as retinyl esters that can be converted to various biological active compounds such as retinoic acid, all-trans retinoic acid and 9-cis retinoic acid (9cRA). 9cRA is the natural ligand for the retinoid X receptor or RXR (NR2B1),[1,2] a ligand-activated transcription factor belonging to the superfamily of nuclear hormone receptors (NHRs). In fact, RXR is a central dimerization partner for several members of this superfamily, including the thyroid hormone receptor (TR), vitamin D receptor (VDR), peroxisome proliferator activated receptors (PPAR's), liver X receptor (LXR), pregnane X receptor (PXR), farnesoid X receptor (FXR) and RXR itself. The ligands for these receptors are metabolites or drugs that, through activation of the NHR, regulate the transcription of genes involved in metabolism and/or transmembrane transport of the ligand. The fact that these NHRs are active as heterodimers with RXR emphasizes the importance of vitamin A for a wide range of physiological processes.

FXR is the mammalian bile acid sensor that controls bile acid homeostasis. Consequently, FXR is crucial for cholesterol metabolism and the intestinal uptake of fat-soluble vitamins. It binds and is activated by bile acids.[3-5]. Together with RXR, it directly stimulates the expression of the hepatocanalicular *bile salt export pump (BSEP),*[6,7] the *ileal bile acid binding protein (IBABP),*[8] *Phospholipid Transfer Protein (PLTP,* [9] and the transcription factor S*mall Heterodimer Partner 1 (SHP).*[10] SHP represses the expression of *cholesterol 7a-hydroxylase,*[11] the rate-limiting enzyme in the bile acid biosynthesis, and the *sodium-dependent taurocholic acid cotransporting peptide (NTCP),*[12] the basolateral bile acid importer of the hepatocyte.

9cRA may exert 3 different effects on transcriptional regulation by NHR/RXR heterodimers; 1) it is a prerequisite for activation but does not activate by itself (non-permissive heterodimers) as observed for RXR/RAR;[13] 2) it activates transcription by itself and has an additive effect on the activation by the heterodimer partner's ligand (permissive heterodimers; RXR/PPAR[14] and RXR/LXR[15]) or 3) it may actually inhibit the transcription activation exerted by the ligand of the heterodimer partner (RXR/VDR).[16,17] FXR/RXR has been described as a permissive heterodimer in the transcriptional control of *I-BABP* and *PLTP.*[8,9]

In this study we show a target-gene selective effect of 9cRA on the FXR/RXR-controlled transcription. *In vitro*, 9cRA strongly antagonized the bile acid-induced transcription of human *BSEP*, while enhancing the transcription of *SHP*. We found that 9cRA exerted this effect on *BSEP* transcription by inhibiting the binding of the FXR/RXR protein complex to the FXR-responsive element in the *BSEP* promoter element. We confirmed these findings in *in vivo* experiments using vitamin A-deficient mice. Cholic acid feeding of these animals resulted in significantly increased *Bsep* expression compared to vitamin A-sufficient mice, whereas S*hp* mRNA levels were highest under conditions of sufficient vitamin A. We discuss our findings in relation to cholestatic disease.

4.3 Materials and methods

Cell culture and transfections

Standard culture conditions for the human hepatoma cell line HepG2-rNtcp and derivatives have been described before.[7] In some experiments, the serum concentration, and thereby the vitamin A/retinol concentration, in the medium was reduced to 1% (vol/vol) as specified in the text. HepG2-rNtcp cells were transfected with various combinations of plasmids (indicated in the text) as described before.[7] Eighteen hours after transfection, medium was refreshed and contained chenodeoxycholic acid (CDCA) and/or 9cRA in various concentrations, as described in the text. Cells were harvested after 24 hours for determination of luciferase activity and after 48 hours for total RNA isolation. HepG2-rNtcp cells stably expressing rat Fxr were obtained after cotransfecting these cells with pCMX-rFxr and pHMR272.[18] Hygromycin B-resistant clones were collected and rFxr-expressing cells were selected by RT-PCR.

Plasmids

Plasmids for overexpression of rat Fxr (pCMX-rFxr), human RXRα (pSG5-hRXRα), and the luciferase reporter vector containing the –105 to –277 base pairs *BSEP* promoter fragment (pGL3-277), control and carrier plasmids (pCMV5 and pGEM-5) were described before.[7]

Luciferase assay

The luciferase reporter assay to measure the *BSEP* promoter activity was described before.[7]

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA isolation and quantitative real-time detection RT-PCR analysis of *BSEP/Bsep*, *SHP/Shp*, Ntcp and *Rxra* mRNA levels were described before.[7,19] Details about primer- and probe-sequences are available at the authors.

Electrophoretic Mobility Shift Assay

Isolation of nuclear extracts from HepG2-rNtcp cells and the protocol for the electrophoretic mobility shift assay have been described before.[20] The FXRE probe contained the IR-1 sequence (underlined) from the *BSEP* promoter: 5'-GATCCCTTAGGGACATTGATCCTTAGG-3', and was labeled with [α-^{32}P]dATP (Amersham, Buckinghamshire, UK) using Klenow polymerase (Promega, Madison, USA).

Animals

Pregnant (two weeks post coitum) C57BL/6J mice were obtained form Harlan Nederland (Horst, the Netherlands). Food and water were available ad libitum. The mice were housed in a temperature-controlled environment with alternating 12 hours

light and dark cycles. Experimental protocols were approved by the Ethical Committee on Animal Testing of the Faculty of Medical Sciences, University of Groningen.

Experimental design

Male C57BL/6J mice were made vitamin A-deficient essentially as described by Smith, 1990.[21] In short, pregnant C57BL/6J mice received a standard laboratory chow upon arrival. At parturition (one week after arrival), the dams were divided into two groups (dam + offspring). One group received a vitamin A-deficient diet (VAD; 4148.10, Hope Farms, Woerden, the Netherlands) and the control group received control diet (4068.02, Hope Farms BV, Woerden, the Netherlands), containing 18,000 IU vitamin A/kg. After weaning (21-25 days), the male pups continued on the same diet as the mother. At the age of 13 weeks, both groups (VAD and control) were divided into two subgroups. Group 1 continued with the VAD diet and group 2 continued with the VAD diet, but now supplemented with 0.5% (wt/wt) cholic acid (CA). Group 3 continued on the control diet and group 4 continued on the control diet, but now diet supplemented with 0.5% (wt/wt) CA. One week later, the mice were weighed and killed. The livers were removed, weighed, cut into pieces, snap-frozen in liquid nitrogen, and stored at -80˚C for mRNA isolation, Western blot analysis and immunofluorescence microscopy. Vitamin A deficiency was determined by high performance liquid chromatography analysis of retinol levels in fresh liver tissue according to Academic Hospital Groningen protocols.

Liver plasma membrane isolation

Total plasma membranes were isolated from 3 to 4 pooled mouse livers using sucrose-gradient ultracentrifugation according to Meier and Boyer, 1990[22] with some modifications as described by Vos et al., 1998.[23]

Western Blot analysis

Equal amounts of liver plasma membrane proteins were separated by 7.5% SDS-PAGE[24] and analyzed for Bsep and Na^+K^+-ATPase protein expression by Western blotting.[25] Polyclonal antibodies used, were raised against Na^+K^+-ATPase (gift from Dr. W. Peters, University Medical Center, Nijmegen, the Netherlands) and BSEP (K12[23] Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as standard.

Confocal laser scanning microscopy

For microscopical analyses, frozen mouse liver tissue was cut into 4 μm-sections. After drying, the liver sections were fixed in acetone and stained for Bsep expression using K12, with the corresponding second antibody Alexa fluor 488 (Alexis Biochemicals, Lausen, Switzerland). Images were taken with a confocal laser scanning microscope (TCS 4D; Leica, Heidelberg, Germany) equipped with an argon/krypton laser and coupled to a Leitz DM IRB (Leica, Heidelberg, Germany) inverted microscope.

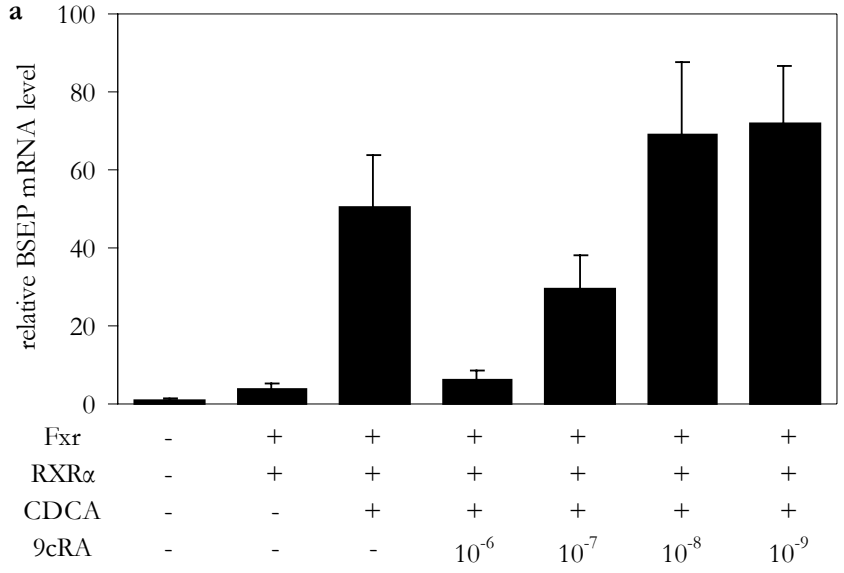
Statistics

Data are presented as means ± sd. Differences between the animal groups were determined in SPSS by one-way ANOVA with Scheffe as post hoc test and $p<0.05$.

4.4 Results

9-cis Retinoic acid antagonizes CDCA-induced expression of human *BSEP* in vitro

To study the role of 9cRA in the transcriptional regulation of the human *BSEP* gene, we transfected HepG2-rNtcp cells with expression vectors for rFxr and hRXRα and cultured these cells in the presence of CDCA with or without various concentrations of 9cRA. As observed before, CDCA alone resulted in a strong (50-fold) increase of *BSEP* mRNA level (Fig. 4-1a). Surprisingly, the bile acid induction was strongly attenuated (to 6.0-fold compared to control cells) when also 1 µmol/L 9cRA was added to the culture medium. This inhibitory effect was dose-dependent, and disappeared when 9cRA concentrations were reduced to 10 nmol/L or lower. Notably, a 9cRA-dependent elevation of *BSEP* expression above the CDCA-induced levels was never observed. The 9cRA-dependent suppression of the CDCA-induced expression of human *BSEP* was also observed in untransfected HepG2-rNtcp cells (Fig. 4-1b). Reducing the concentration of serum, which also contains 9cRA/vitamin A (± 0.5 µmol retinol/L), in the culture medium to 1% strongly increased the CDCA-induced expression of *BSEP*, which was suppressed after adding 9cRA to this medium. In HepG2-rNtcp cells stably transfected with rat Fxr, the CDCA and 9cRA regulated expression of endogenous *BSEP* was similar, but strongly amplified compared to untransfected HepG2-rNtcp cells (Fig. 4-1c).



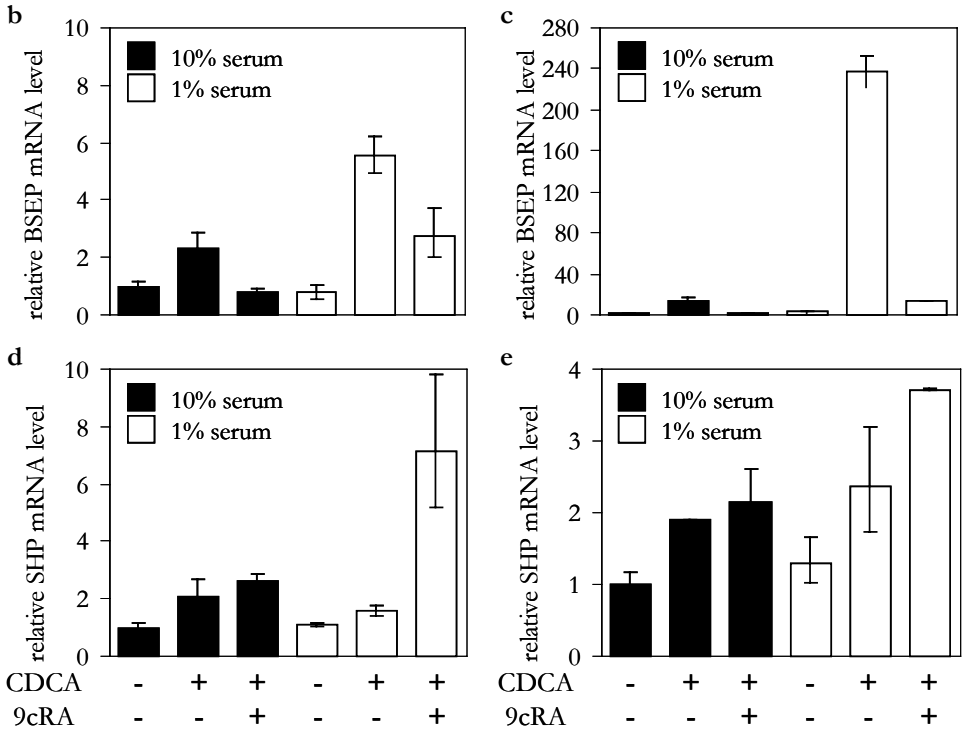


Fig. 4-19cRA inhibits CDCA-stimulated *BSEP* mRNA expression. (a) *BSEP* mRNA levels in HepG2-rNtcp cells after transfection with the indicated expression vectors for hRXRα and/or rFxr, and treatment with 100 μmol/L CDCA with or without simultaneous addition of 9cRA in the concentration range of 1μmol/L to 1 nmol/L. *BSEP* (b,c) and *SHP* (d,e) mRNA levels in HepG2-rNtcp cells (b,d) or HepG2-rNtcp cells stably expressing rFxr (c,e), cultured in 10% (vol/vol) serum (black bars) or 1% serum (vol/vol) (white bars), and incubated with 50 μmol/L CDCA or 1 μmol/L 9cRA as indicated. *BSEP* and *SHP* relative mRNA levels were determined by RT-PCR and normalized to *18S*. Values are presented as mean ± sd.

9cRA agonizes CDCA-induced expression of human *SHP*

The small heterodimer partner 1 (*SHP*) is also a well-established FXR target gene. Therefore, we determined the effect of 9cRA on the CDCA-induced expression of *SHP*. In untransfected HepG2-rNtcp cells, CDCA-induced expression of *SHP* 2.0-fold and 9cRA further increased this to 2.6-fold relative to control cells (Fig. 4-1d). The additive effect was also observed when these cells were grown in medium containing 1% serum and in HepG2-rNtcp cells stably expressing rFxr (Fig. 4-1e). Thus, 9cRA agonizes CDCA-induced expression of *SHP*, while it antagonizes *BSEP* expression.

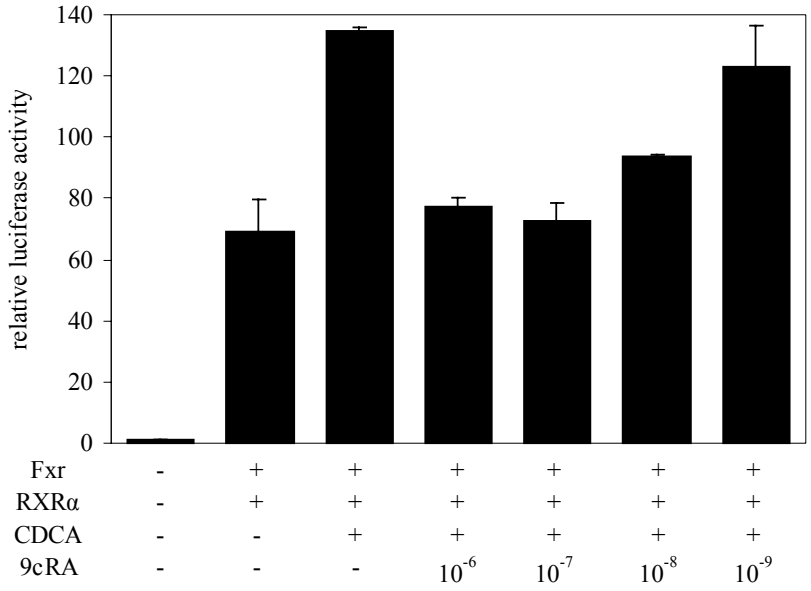


Fig. 4-2 The CDCA-induced promoter activity of *BSEP* is antagonized by 9cRA. HepG2-rNtcp cells were transfected with pGL3-277 in combination with the indicated expression vectors for hRXRα and/or rFxr, and were treated with 100 μmol/L CDCA with or without simultaneous addition of various concentrations of 9cRA (indicated as mol/L). After 24 hours, cells were harvested and luciferase activities were determined. Values are presented as mean ± sd.

9cRA reduces the CDCA-induced *BSEP* promoter activity

Luciferase reporter gene assays were performed to determine whether 9cRA acts directly on the *BSEP* promoter activity. HepG2-rNtcp cells were transfected with pGL3-277, containing the minimal *BSEP* promoter sequence including the FXR responsive element (FXRE), in combination with rFxr and hRXRα expression plasmids. When grown in the absence of CDCA and 9cRA, these cells showed significant levels of luciferase activity (69-fold induced compared to pGL3-227 alone), probably due to stimulation of the rFxr/hRXRα by bile acids produced by HepG2 cells.[26,27]. Luciferase activity was, however, super-induced by the addition of CDCA to the medium (134-fold). As observed with the endogenous *BSEP* mRNA levels, 1 μmol/L 9cRA fully inhibited the CDCA-induced luciferase activity and this effect was also dose-dependent (Fig. 4-2).

9cRA reduces FXR/RXR binding to the *BSEP*-FXR responsive element

Next, we studied whether 9cRA caused changes in the binding of the FXR/RXR heterodimer to the FXRE in the *BSEP* promoter. An electrophoretic mobility shift assay was performed using the human *BSEP* FXRE sequence and nuclear extracts

from HepG2-rNtcp cells cultured in the absence or presence of CDCA and 9cRA. In control cells (Fig. 4-3, lanes 1 and 2) and cells treated with CDCA, a clear band representing the FXR/RXR heterodimer was observed (Fig. 4-3, lane 3 and 4). In cells treated with CDCA and 9cRA this band is absent (Fig. 4-3, lanes 5 and 6), showing that 9cRA reduces binding of the endogenous human FXR/RXR heterodimer to the FXRE target sequence in the human *BSEP* promoter.

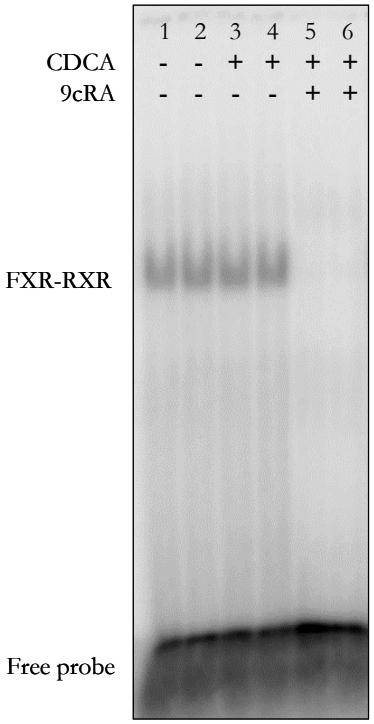


Fig. 4-3 FXR/RXR binding to the *BSEP*-FXRE oligonucleotide is lost upon 9cRA treatment. HepG2-rNtcp cells were incubated with vehicle (lanes 1 and 2), with 100 μmol/L CDCA (lanes 3 and 4), or with 100 μmol/L CDCA and 1 μmol/L 9cRA (lanes 5 and 6). Nuclear extracts were obtained and an electromobility shift assay was performed using the radioactively labeled FXRE from the *BSEP* promoter.

Vitamin A deficiency increases cholic acid-induced Bsep expression in vivo

To establish the role of vitamin A, and its derivative 9cRA, in regulating BSEP levels *in vivo*, we analyzed the effect of cholic acid-feeding in normal and vitamin A-deficient mice. From birth, male C57BL/6J mice were either fed a vitamin A-sufficient (control) or a vitamin A-deficient (VAD) diet. At 13 weeks of age, both groups were subdivided in two: one group continued on the same diet, while in the other group the diet was supplemented with 0.5% (wt/wt) CA. One week later, the mice were killed and analyzed for hepatic gene expression.

Survival of control mice was 100%, while the survival of the VAD mice was 50% (without CA) and 54% (with CA) (Table 4-1). The survivors in the VAD groups appeared healthy and showed no apparent symptoms of deficiency.
Retinol levels were undetectable in the livers (Table 4-1) and serum (data not shown) from VAD mice, whereas they were readily detectable in mice on control diets. No statistically significant differences were observed between control and VAD mice for general parameters, like body weight, liver weight, and liver to body ratio (Table 4-1), except for the mean body weight of the VAD group ($p = 0.03$ versus control) and the VAD with CA diet group ($p = 0.012$ versus VAD). The plasma bile salts of the VAD with CA diet group was significantly different compared to the control group ($p = 0.018$) and the VAD group ($p = 0.026$).

	Control n=6 Mean ± sd	**Control+CA** n=7 Mean ± sd	**VAD** n=12 Mean ± sd	**VAD+CA** n=13 Mean ± sd
Body weight (g)	26.9±1.1	25.8±.6	22.9±2.8[a]	26.6±1.1[c]
Liver weight (g)	1.0±0.2	1.2±0.1	1.0±0.2	1.1±0.2
Liver to body ratio	0.037±0.006	0.045±0.003	0.044±0.005	0.042±0.007
Plasma bile salts (µmol/L)	36.5±6.9	62.3±10.8	40.3±7.9	76.4±34.1[ac]
Survival (%)	100	100	50	54
Liver vitamin A (µmol/g)	93.6±33.8	135.9±34.2	n.d.	n.d.

Table 4-1 Animal characteristics, hepatic vitamin A level and survival percentage in male C57BL/6J mice, either fed the control or VAD diet, with or without 0.5% (wt/wt) CA supplementation. Data are presented as mean ± sd. (a: significantly different from control group; c: significantly different from VAD group; n.d.: not detectable)

Hepatic *Bsep*, *Shp*, *Ntcp* and *Rxra* mRNA expression was determined by quantitative real-time RT-PCR (Fig. 4-4) and Northern blot analysis (*Bsep*, data not shown). Both methods revealed that CA-feeding of VAD mice resulted in highest *Bsep* mRNA levels (3.4-fold increased compared to control ($p < 0.001$) whereas it led to only a 1.7-fold increase in mice on control diets (Fig. 4-4a). *Bsep* mRNA levels were not increased in mice receiving the VAD diet without CA. In contrast, *Shp* levels were highest in cholic acid-fed control mice (2.2-fold increase compared to control), slightly reduced in VAD mice (0.7-fold) but still inducible by CA-feeding (1.7-fold) (Fig. 4-4b). This was also reflected in the *Ntcp* mRNA levels that were lowest in CA-fed control mice (0.3-fold, $p = 0.007$ versus control) and not significantly changed in VAD mice (Fig. 4-4c). Importantly, *Rxra* expression was similar in all 4 groups, with only a slight, although significant, increase in the VAD+CA group (1.5-fold increase compared to control, $p = 0.007$, Fig. 4-4d).

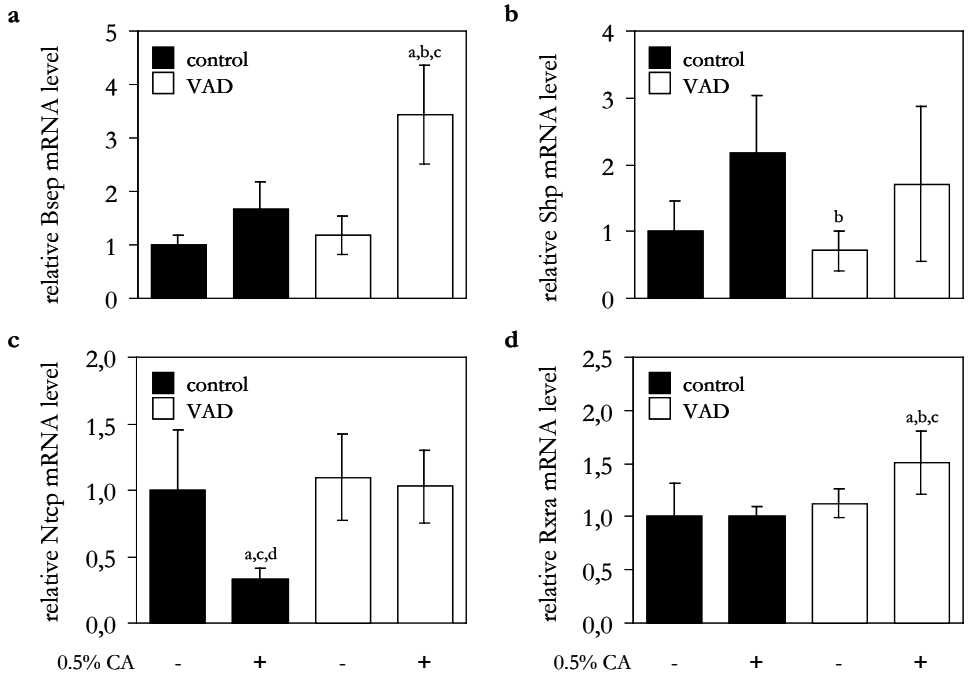


Fig. 4-4 Bsep mRNA expression is significantly increased by cholic acid-feeding in VAD mice, but not in control mice. Total RNA was isolated from liver sections of mice either fed the control diet (black bars) or VAD diet (white bars), with (+) or without (-) 0.5% (wt/wt) CA supplementation. Relative Bsep (a), Shp (b), Ntcp (c) and Rxrα (d) mRNA levels normalized to 18S were analyzed by RT-PCR. Data are presented as mean ± sd of individual mice per group. (a: significantly different from control group; b: significantly different from control+CA group; c: significantly different from VAD group; d: significantly different from VAD+CA group)

The difference in *Bsep* mRNA levels was also reflected in the Bsep protein level in liver membrane fractions (Fig. 4-5). As shown for the *Bsep* mRNA, CA-feeding in control mice and vitamin A deficiency by itself did not result in altered Bsep protein levels (Fig. 4-5). In contrast, Bsep protein levels were significantly increased in VAD mice fed a CA-containing diet (Fig. 4-5).

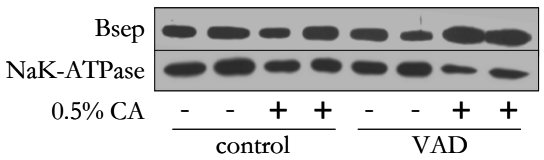


Fig. 4-5 Bsep protein expression is increased by cholic acid-feeding in VAD mice, but not in control mice. Liver plasma membranes were isolated from pools of 3 to 4 livers from mice either fed the control of VAD, supplemented with (+) or without (-) 0.5% (wt/wt) CA. Equal amounts of two independent membrane isolations were subjected to Western blot analysis, and stained for Bsep and Na+K+ATPase.

Immunofluorescence microscopical analyses of liver sections revealed a normal canalicular staining for Bsep for mice from all 4 groups, indicating that the VAD diet did not give rise to abnormal Bsep sorting in hepatocytes (Fig. 4-6).

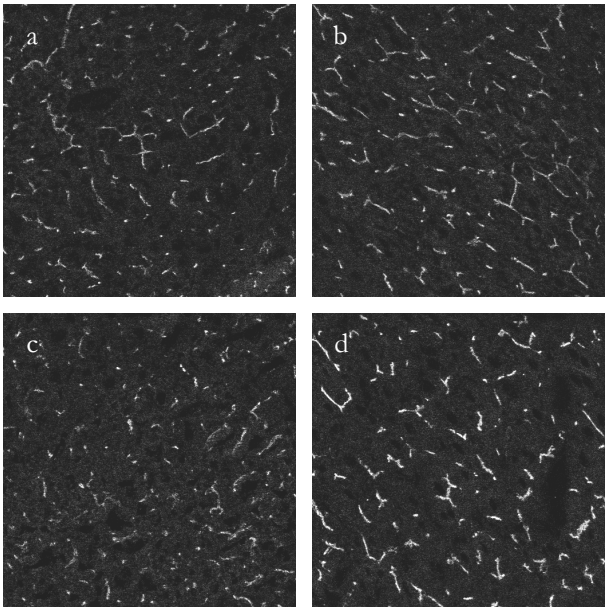


Fig. 4-6 Bsep protein expression shows a normal canalicular staining pattern in VAD mice. Liver sections from mice, fed the control diet (a), control diet supplemented with 0.5% (wt/wt) CA (b), VAD diet (c) or VAD diet supplemented with 0.5% (wt/wt) CA (d), were stained for Bsep. The protein expression pattern was analyzed using confocal laser scanning microscopy.

4.5 Discussion

In this study, we show that vitamin A and its derivative 9cRA play an important role in the transcriptional regulation of genes controlling bile acid homeostasis. *In vitro*, 9cRA antagonized the bile acid-induced expression of *BSEP* by the FXR/RXR heterodimer, while it agonized *SHP* expression. *In vivo*, vitamin A deficiency strongly increased the bile acid-induced *Bsep* mRNA and protein levels in CA-fed mice.

BSEP is specifically expressed in the liver and transports bile acids across the canalicular membrane.[28] It plays a crucial role in the enterohepatic circulation of bile acids.[29,30]. Previous studies have shown that *BSEP* gene transcription is controlled by its substrates, mediated through the bile acid sensor FXR and its heterodimer partner, RXR.[6,7]. Other FXR/RXR target genes include *SHP,*[10] *IBABP*,[8] *PLTP*,[9] *Dehydroepiandrosterone Sulfotransferase*,[31] *Apolipoprotein C-II*,[32] *Multidrug Resistance-Associated Protein 2*,[33] *Organic Anion Transporting Polypeptide 8*,[34] *Kininogen*,[35] *Bile acid-CoA synthetase* and *Bile acid-CoA: amino acid N-acetyltransferase*.[36] Expression analyses on *IBABP* and *PLTP* have shown that maximal FXR/RXR transcriptional activation was observed when both FXR- (CDCA) and RXR- (9cRA) ligands were present.[8,9] This study

revealed similar results for *SHP*, but the opposite effect was observed for *BSEP*. 9cRA strongly attenuated the bile acid-induced *BSEP* transcription by FXR/RXR.
One other example of an inhibitory effect of 9cRA on an RXR/NHR heterodimer has been described and studied in detail. In concert with RXR, the vitamin D receptor (VDR) regulates the transcription of genes involved in calcium and phosphate homeostasis.[37] Transcription of the rat osteocalcin gene was found to be activated by VDR, RXR and the VDR-ligand 1,25-dihydroxyvitamin D3, but inhibited by 9cRA, which destabilized the DNA bound RXR/VDR heterodimer.[16] Similarly, we found that the presence of 9cRA resulted in a decreased formation of the FXR/RXR-DNA complex as shown by electrophoretic mobility shift assay analysis. Our data are in line with the recent report by Kassam et al.,[38] who showed that the RXR ligands LG100268 and 9cRA inhibit the binding of *in vitro* translated FXR and RXR to the *BSEP*-FXRE. In addition, we show that 9cRA is required for maximal CDCA-induced expression of *SHP*. The molecular basis for the variable effect of 9cRA on FXR/RXR target genes is still unclear, but may be caused by differences in or close to the FXRE in the corresponding promoter elements or the selective interaction with transcription coactivators.[38]
The crucial question we wanted to address in this study was: does vitamin A play a role in *BSEP* expression *in vivo*? From the *in vitro* data we hypothesized that physiological levels of vitamin A may actually reduce bile acid-induced expression of *Bsep* in mice with a normal vitamin A status. In line with this, Wolters et al.[39] reported only minor effects on Bsep expression after taurocholic acid-feeding of wild type mice. Therefore, we fed C57BL/6J mice a vitamin A-deficient diet and analyzed its effect on mRNA levels of the Fxr-target genes *Bsep* and *Shp*. Vitamin A deficiency in mice strongly increased bile acid-dependent *Bsep* expression, whereas it reduced *Shp* expression, confirming our *in vitro* results using human HepG2 cells.
These data may have important implications for therapeutic interventions aimed at regulating canalicular bile acid secretion in cholestatic patients. Chronic liver disease, in particular chronic cholestatic liver disease, leads to vitamin A deficiency.[40,41] However, vitamin A supplementation in liver disease is a controversial issue.[42] Our data may explain this phenomenon. In chronic cholestatic liver disease, the increased intrahepatocellular bile acid concentration, in combination with a decreased vitamin A concentration, presents the optimal condition for *BSEP* induction. *NTCP*, however, remains expressed because of a lack of induction of *SHP*, the negative regulator of *NTCP*. Thus the hepatobiliary secretion of bile acids remains intact to some extend. Supplementation with vitamin A would, in theory, aggravate the cholestasis in these patients: it would reduce *BSEP* expression, activate *SHP* and thereby reduce *NTCP* expression. When viewed from the hepatocyte, vitamin A has a cytoprotective effect. However, in case of extrahepatic bile duct obstruction, persistent or elevated BSEP activity may contribute to increase intraluminal pressure in the intrahepatic cholangioles. This may result in bile infarcts and cause considerable damage of the liver parenchyma.[43] Ursodeoxycholic acid therapy may be harmful for these patients, since it further increases biliary pressure. Reducing BSEP expression through vitamin A supplementation may protect the liver in this case. Support for this hypothesis was recently provided by de Freitas et al., who showed that vitamin A supplementation in

combination with biliary obstruction results in a significant reduction of liver fibrosis.[44]

In conclusion, it is evident that vitamin A plays a crucial, but complex role in the physiological processes regulated by the nuclear hormone receptors. Vitamin A is required for optimal activation of RXR/RAR-, RXR/TR-, RXR/LXR- and RXR/PPAR-target genes. In contrast, high levels of vitamin A attenuate, and low levels stimulate, bile acid-induced transcription of *BSEP*. These observations show that 9cRA is an important molecular switch and that its effect needs to be determined for every individual RXR/NHR target gene and cannot be extrapolated from data obtained from studies on other target genes. Finally, the clinical role of vitamin A repletion in chronic liver disease needs to be redefined.

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